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Presence of Two Distinct Transcripts for Matrilysin in Porcine Ovary

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ABSTRACT—A cDNA encoding a matrix metalloproteinase-related protein has been cloned from a porcine ovary cDNA library. This putative protein comprises 119 amino acid residues, and exhibits sequence similarity with the NH_2-terminal half of rat matrilysin with 264 amino acid residues. A porcine ovary matrilysin cDNA clone was also isolated and characterized. A structural comparison of the matrilysin-related protein with porcine matrilysin suggests that both proteins are the products of the same gene. To further clarify their relationship, PCR amplification was carried out using porcine genomic DNA with two appropriate primers. The sequence analysis of a resulting 3.5kb PCR product revealed that a processing step involving internal exons of the gene produces the matrilysin-related protein. Relative amounts of the two distinct mRNAs in the porcine ovary were estimated by the competitive PCR method. The levels of mRNA for the matrilysin-related protein were approximately 4% of the wild-type matrilysin. The results indicate that the matrilysin-related protein is specifically expressed in the porcine ovary.

INTRODUCTION

The extracellular matrix (ECM) is important in supporting tissue structures, and its individual components are solely and/or cooperatively involved in various cellular events. Degradation of the ECM is known to occur during a variety of tissue-remodeling processes, including tumor invasion and metastasis (Garbisa *et al*., 1980; Kohn and Liotta, 1995; Mignatti and Rifkin, 1993), ovulation (Tsafriri, 1995; Reich *et al*., 1985), embryonic growth and differentiation (Sappino *et al*., 1989), and the development of organs (Raponen *et al*., 1994). These processes require the concerted action of a number of extracellular proteinases. Among these, matrix metalloproteinases (MMPs) are thought to play crucial roles (Woessner, 1991; Werb *et al*., 1996; Werb, 1997; Chambers and Martrisian, 1997).

The MMP family is composed of at least 24 members with different, albeit overlapping, substrate specificities (Hulboy *et al*., 1997; Nagase and Woessner, 1999; Pei, 1999a; Llano et al., 1999). Regarding their involvement in the biological processes associated with mammalian reproductive organs, the smallest MMP, matrilysin (EC 3.4.24.23; also known as MMP-7) has been studied the most extensively. Such studies clearly indicate that this enzyme is important for the function of the human (Rodgers *et al*., 1993) and rat (Abramson *et al*., 1995; Woessner, 1996) and mouse uterus (Rudolph-Owen LA *et al*., 1997) as well as the mouse mammary gland (Rudolph-Owen LA *et al*., 1998) and male reproductive tract (Wilson *et al*., 1995; Rudolph-Owen LA *et al*., 1998). The biological role of MMPs in the ovary is not yet fully understood although there have been several reports documenting the activities and gene expressions of some enzymes belonging to this family in relation to follicular development and ovulation (Murdoch and McCormick, 1992; Tadakuma *et al*., 1993; Butler *et al*., 1991; Liu *et al*., 1998). In this regard, a new type of MMP (MMP-21/MMP-22/MMP-23/CA-MMP), which is predominantly expressed in mammalian reproductive organs, such as ovary and uterus, has been recently discovered (Gururajan *et al*., 1998a; Gururajan *et al*., 1998b; Velasco *et al*., 1999; Pei, 1999b). This novel MMP is a membrane-associated enzyme capable of degrading gelatin (Pei, 1999b), and lacks a classic cysteine switch but possesses two distinct motifs: a cysteine array and a Ig-fold.

We are interested in determining the role of MMPs linked to ovarian function. In our preliminary experiments searching for MMPs that are expressed in mammalian ovaries, we happened to find a novel cDNA clone from the porcine ovary cDNA library. The present study was therefore undertaken to examine this clone and to clarify its relationship with known MMP species. Our data have revealed that this unique clone is derived from the same matrilysin gene as a result of different splicing of mRNA. This novel transcript can be detected in the porcine ovary but not in the testis and liver.

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MATERIALS AND METHODS

Materials

Porcine ovaries were obtained from a local slaughterhouse within

30 min of the animals' death and transported to the laboratory on ice.

Isolation of a matrilysin-related cDNA from a porcine ovary cDNA library

A rat matrilysin cDNA fragment was amplified by reverse-transcriptase polymerase chain reaction (RT-PCR) from rat ovary total RNA using an oligo(dT) primer. A sense primer of 5'-CAGGCG-CAGAATTATCTTAGG-3' corresponding to nucleotides 142–162 and an antisense primer of 5'-GCATACTCCACATTCTGAGAGC-3' complementary to the nucleotides 436–457 of rat matrilysin cDNA (Abramson *et al.*, 1995) were used. For the reaction, 1 μg of poly(A)$^+$RNA was reverse-transcribed, and the resulting cDNA was subjected to 35 cycles of PCR using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) under the following conditions: 97°C for 30 sec, 56°C for 15 sec, and 72°C for 2 min. The PCR product was used as a probe for screening a matrilysin cDNA clone.

Total RNA was prepared from porcine ovary by the guanidine isothiocyanate-cesium chloride method (Chirgwin *et al.*, 1979). Poly(A)$^+$RNA was selected by oligo(dT)-cellulose column chromatography and used for the synthesis of cDNA by the cDNA Synthesis Module (Amersham Pharmacia Biotech, Tokyo, Japan). The cDNA larger than 0.4 kb in length was inserted into λgt10-*Eco*RI arm (Takara, Tokyo, Japan) and packaged with GIGAPACK II GOLD (Stratagene). The 2.5×10^5 plaques were screened by plaque hybridization using labeled probe described above. The probe was labeled with [^{32}P]-dCTP using the Random Primer DNA Labeling Kit Ver. 2 (Takara). A positive clone was isolated and sequenced using ABI automatic sequencer model 377 (Perkin-Elmer/Applied Biosystems).

cDNA cloning of porcine matrilysin by the RT-PCR method

A normal type of matrilysin was amplified by RT-PCR from porcine ovary using a sense primer S3, 5'-CAGCAGCTATGCAGCT-3', and an antisense primer AS4, 5'-CAGAGCGGGAGAGAGACATAT-3'. One microgram of poly(A)$^+$RNA was reverse-transcribed with the Superscript Preamplification System (Gibco BRL, Rockville, MD) and one-twentieth of the reaction product was used as a template for the PCR using *Pfu* DNA polymerase (Stratagene). The PCR was performed for 35 cycles under the following conditions: 96°C for 30 sec, 57°C for 30 sec, and 72°C for 2 min.

Amplification of the porcine matrilysin gene

A genomic clone of porcine matrilysin was amplified from genomic DNA. The PCR was performed using a sense primer S1, 5'-TACTGTGTGCTGTGTGTCTGC-3', and an antisense primer AS1, 5'-CTGTGACATGTGGTAGGTCTCG-3', under the following conditions: 35 cycles of 96°C for 30 sec, 60°C for 30 sec, and 72°C for 2 min. A major band of 3.5 kb was isolated and sequenced.

Competitive PCR

Competitive PCR was conducted essentially according to the method of Dessi *et al.* (1997). To examine the expression levels of matrilysin and the related protein, two competitive templates were prepared. Both of the full-length cDNAs were digested with *Xho*I and *Sac*I sites, which are located at positions 436 and 572, respectively, to delete a 136 bp fragment. After blunting, the resulting 5'- and 3'-fragment were ligated and used as templates for PCR amplification. For matrilysin, a competitor of 456 nucleotides was amplified with a combination of two primers (a sense primer S5, 5'-GTCACCTA-CAGGATTGTGC-3; an antisense primer AS3, 5'-GGCAGTGAG-TGCCTAGTGAA-3). For the matrilysin-related protein, a 522-nucleotide competitor was prepared by PCR using a sense primer S4 (5'-GCTTTGGCTCATTTAGGC-3') and the same antisense primer AS3. The sequences of competitor templates were confirmed as described above.

The mixture (20 μl in total volume) contained 1 μg cDNA, which had been synthesized with poly(A)$^+$RNA isolated from porcine ovary, various concentrations of competitors, ExTaq polymerase (Takara), and 0.2 μM of primers; this mixture was subjected to PCR using a step program (94°C for 30 sec, 60°C for 30 sec, and 72°C for 90 sec), followed by a 7-min final extension at 72°C. After PCR, the products were separated by electrophoresis on 2.5% agarose gel. The separated products on the gel were blotted onto a nylon membrane in 0.4 N NaOH. The membrane was hybridized with a [^{32}P]-labeled matrilysin cDNA. The radioactive products were visualized using a Bioimage analyzer (Fuji Film, Tokyo). Quantification was performed based on the intensity of the visualized bands.

RESULTS AND DISCUSSION

cDNA cloning of matrilysin-related protein

In order to isolate a cDNA encoding porcine matrilysin, we screened approximately 2.5×10^5 plaques using the rat matrilysin cDNA fragment (316 bp) as described in MATERIALS AND METHODS section. A positive clone with an insert of 1.2 kb was obtained. The corresponding insert was subcloned, and its complete nucleotide sequence was determined. Computer analysis of the obtained sequence using MacVector (Ver. 6.01, Oxford Molecular Ltd.) revealed an open reading frame 357 bp long, starting with an ATG codon at position 9 and ending with a TGA codon at position 368 (Fig. 1). Assuming that translation starts at this first ATG, the identified open reading frame codes for a protein of 119 amino acids, which is half the size of that reported for rat uterus matrilysin (Abramson *et al.*, 1995). However, the overall sequence was highly homologous to that of the NH_2-terminal amino acid sequence of the rat protein: 70 amino acid residues out of 119 residues were shared. The sequence contained the cysteine switch motif, one of the functional domain characteristic of MMPs, but not the zinc-binding region, another functional domain.

Further comparison with porcine matrilysin

Our attempt to isolate a porcine matrilysin cDNA clone by screening the same ovary cDNA library was not successful. As will be described below, the matrilysin transcript content is expected to be much greater than that of the matrilysin-related protein in the mRNA fraction used. Therefore, failure to isolate a relevant cDNA clone is somewhat strange. We have no clear explanation for this at present. RT-PCR method was adopted to amplify porcine matrilysin DNA using the ovary poly(A)$^+$RNA as described in MATERIALS AND METHODS section. The PCR product was subjected to nucleotide sequence analysis, and the results are shown in Fig. 2. The cDNA is 968 bp in length, with an open reading frame of 801 bp and 5'- and 3'-untranslated regions of 8 and 159 bp, respectively. The open reading frame encodes for a protein of 267 amino acid residues. It should be noted that the nucleotide sequences corresponding to S3 and AS4 may not be correct since these were primers used for RT-PCR. In analogy to rat matrilysin (Abramson *et al.*, 1995), we tentatively presume that the signal peptide is cleaved at the peptide bond of Ala^{17}–Leu^{18}. The cysteine switch motif and the zinc-binding region are present in the sequence. Figure 3 shows the amino acid sequences of the matrilysin-related protein and of por-

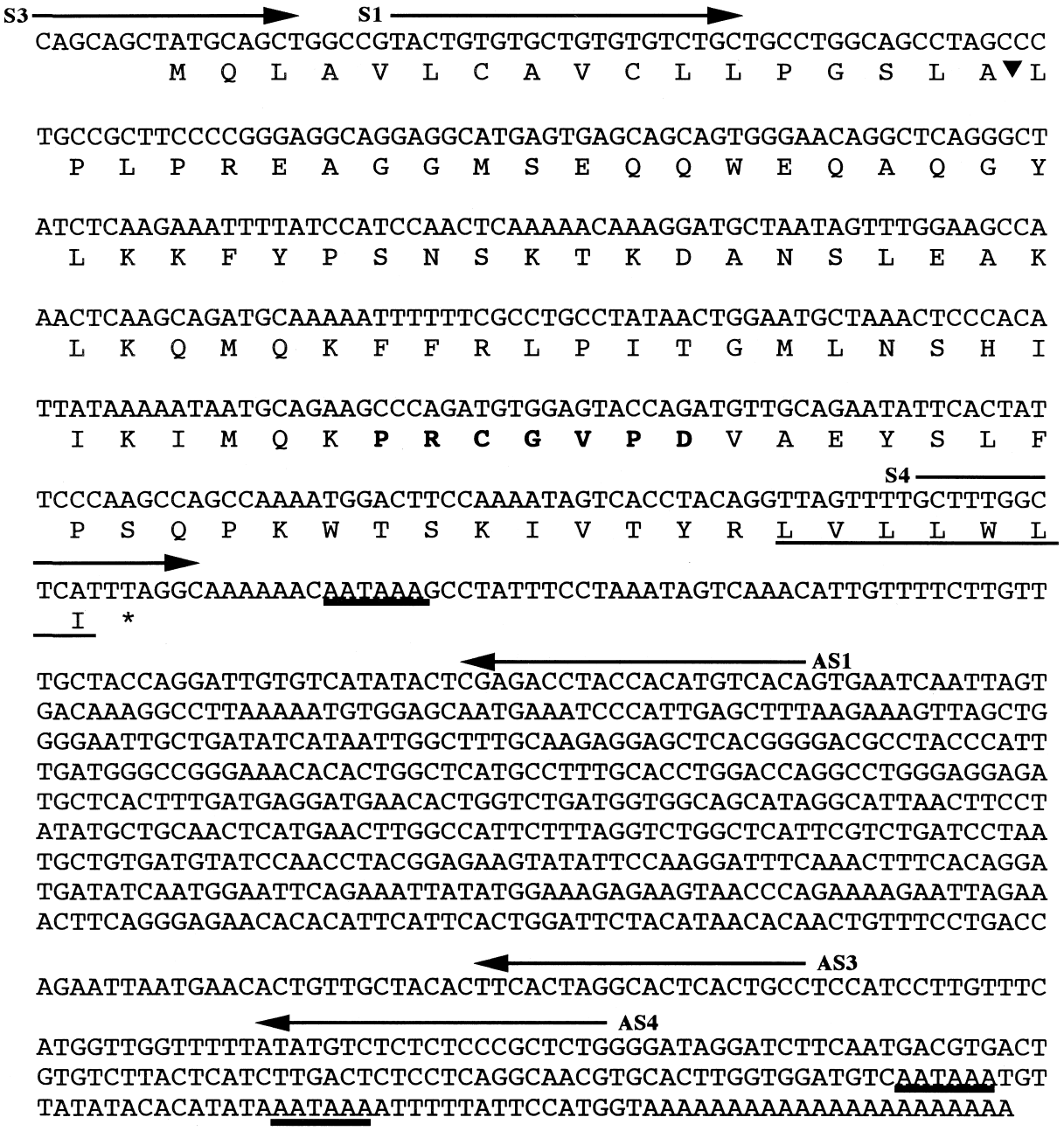

Fig. 1. Nucleotide sequence of the porcine ovary matrilysin-related protein cDNA and its deduced amino acid sequence. The amino acid sequence is shown in lower line and the cDNA sequence in upper line, with numbering on the left. The predicted cleavage site of the signal peptide is indicated by an arrowhead. The cysteine switch-like sequence is shown in **bold**. The COOH-terminal hydrophobic amino acid sequence is underlined. The stop codon (asterisk) and the potential poly(A) addition signal sequences (thick underline) are indicated. The sense and antisense primers used for further experiments are shown (see the text for details). This nucleotide sequence has been submitted to the DDBJ/EMBL/GenBank™ Data Bank with the accession number AB031324.

cine and rat matrilysin, for comparison. The amino acid sequences of rat and porcine matrilysin in the prepro-form are 67.8% identical. As is clear from Fig. 3, the amino acid sequence of porcine matrilysin-related protein is in perfect agreement with the porcine matrilysin sequence for the residues 1–112. These results strongly suggest that the porcine matrilysin-related protein is a product of the porcine matrilysin gene itself. In addition, the nucleotide sequence, from guanine-344 to guanine-968, in Fig. 2 was found to correspond to that from guanine-430 to guanine-1054 in Fig. 1. These comparative analyses clearly indicate that the cDNA for the matrilysin-related protein contains an insertion of 86 nucleotides at around the nucleotide 344 of the porcine matrilysin cDNA.

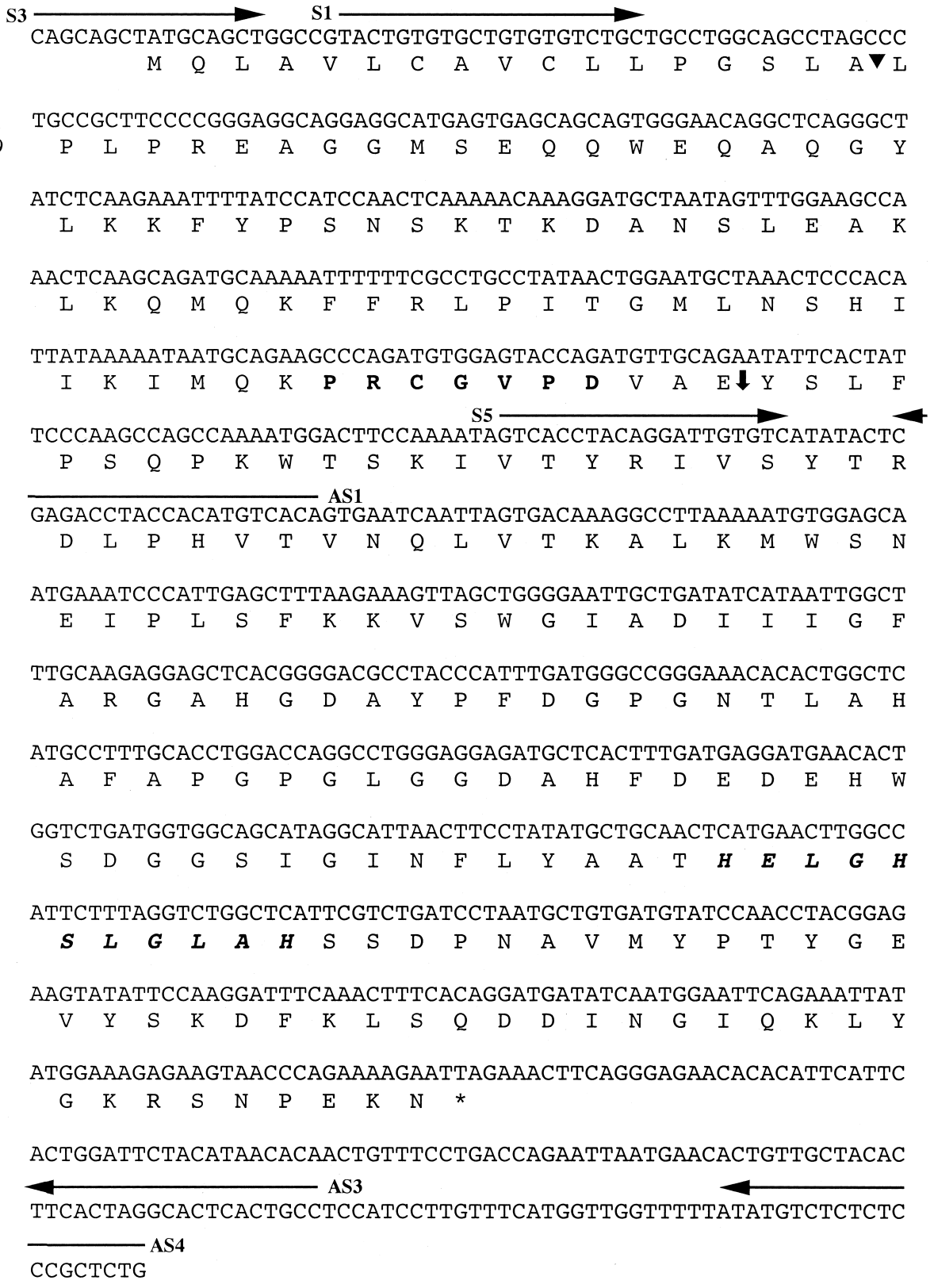

Fig. 2. Nucleotide sequence of the porcine ovary matrilysin cDNA and its deduced amino acid sequence. The predicted cleavage site (arrowhead) of the signal peptide and the activation site (vertical arrow) are indicated. The cysteine switch sequence (**bold**) and zinc-binding region (***italic***) are also shown. The stop codon is indicated by an asterisk. The sense and antisense primers used for further experiments are shown (see the text for details). This nucleotide sequence has been submitted to the DDBJ/EMBL/GenBank™ Data Bank with the accession number AB031323.

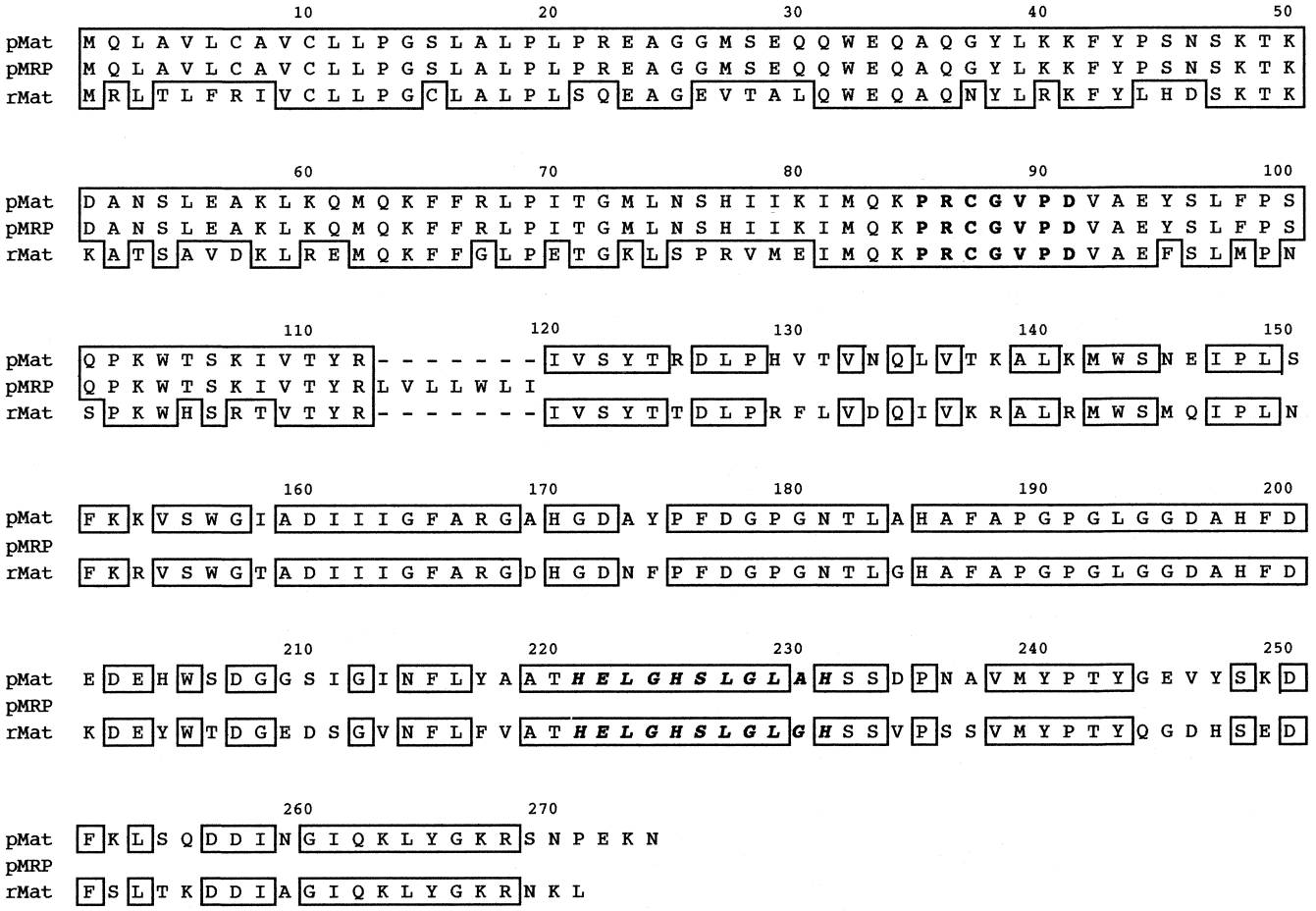

Fig. 3. Homology of porcine matrilysin with the matrilysin-related protein.
Amino acid sequences of porcine matrilysin (pMat), the matrilysin-related protein (MRP), and rat matrilysin (rMat) are aligned. The cysteine switch sequences (Pro[85] to Asp[91]) are shown in **bold** and the zinc-binding regions (His[221] to His[231]) in ***italic***.

Demonstration of the insertion of an intron in the cDNA coding for the matrilysin-related protein

The results described above suggest that the clone for the matrilysin-related protein originates from alternative processing events involving internal exons of porcine matrilysin gene rather than from the unwanted artificial recombination of the cDNA insert during cDNA library construction. To confirm this hypothesis, we conducted PCR amplification experiments using porcine genomic DNA with a set of primers (a sense primer S1, 5'- TACTGTGTGCTGTGTGTCTGC-3' corresponding to nucleotides 22–42, and an antisense primer AS1, 5'-TGTGACATGTGGTAGGTCTCG-3' complementary to the nucleotides 446–466, see Fig. 1 for the nucleotide numbers). A 3.5-kb PCR product was isolated and its sequence analysis identified the same sequence of 86 nucleotides that was found in the cDNA clone of the matrilysin-related protein. As far as the current partial nucleotide sequencing data are concerned, the exon-intron organization of the porcine matrilysin gene is similar to those previously reported for the human (Gaire *et al.*, 1994) and mouse matrilysin genes (Wilson *et al.*, 1995). Based on these considerations, we tentatively conclude that the 86 nucleotides inserted in the novel cDNA represent intron 2 of the porcine matrilysin gene (Fig. 4).

Differential detection of the two mRNAs transcribed from the porcine matrilysin gene

An experiment to detect the two distinct mRNAs by RT-PCR was carried out using porcine ovary, testis, and liver poly(A)$^+$RNA. To this end, we used two sets of primers as follows: a sense primer S5 (5'-GTCACCTACAGGATTGTGTC-3' corresponding to nucleotides 333–352 in Fig. 2) and an antisense primer AS3 (5'-GGCAGTGAGTGCCTAGTGAA-3' complementary to nucleotides 901-920 in Fig. 2) for porcine matrilysin, and a sense primer S4 (5'-GCTTTGGCTCA-TTTAGGC-3' corresponding to nucleotides 353–370 in Fig. 1) and the same antisense primer AS3 (5'-GGCAGTGAGT-GCCTAGTGAA-3' complementary to nucleotides 987–1006 in Fig. 1) for the matrilysin-related protein. As shown in Fig. 5, two PCR products of the size expected were amplified with ovary poly(A)$^+$RNA. When the liver poly(A)$^+$RNA was used, only a product corresponding to the porcine matrilysin was produced. No products were detected in the testis. These results indicate that the mRNA for the matrilysin-related pro-

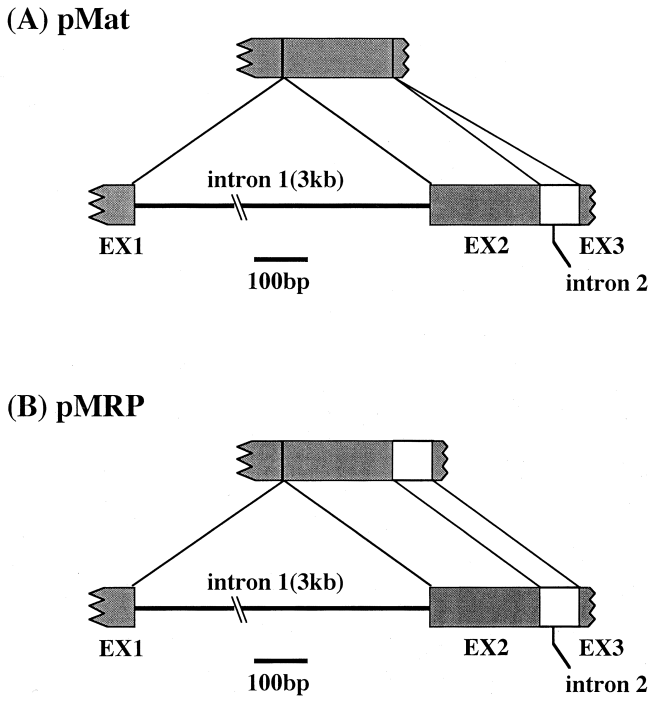

Fig. 4. Schematic representation of the partial structure of porcine ovary matrilysin and the related protein mRNA generated from the matrilysin gene by alternative RNA splicing. A 3.5-kb fragment of the porcine matrilysin gene was obtained by PCR. This fragment contained an 86-nucleotide sequence designated as intron 2 in the figure. The 86-nucleotide sequence is present in the matrilysin-related protein mRNA (B) but not in the matrilysin mRNA (A).

tein is synthesized quite specifically in the ovary, although its tissue distribution in other organs remains to be examined. Regarding the matrilysin mRNA expression in mice, Wilson *et al.* (1995) reported that the transcript was not detected with these tissues even by RT-PCR. Obviously, there are some differences in expression between the two species.

We next determined the expression level of the matrilysin-related protein mRNA relative to that of the wild-type matrilysin by competitive PCR in the ovary. Using the same combinations of primers as in Fig. 5, the two mRNAs were specifically and quantitatively amplified (Fig. 6). Based on these results, the mRNA amounts of porcine matrilysin and the matrilysin-related protein were estimated to be approximately 0.25 and 0.01 pg/μg poly(A)$^+$RNA, respectively. Therefore, the percentage of the unusual transcript for matrilysin relative to that for the wild type matrilysin was only about 4%.

In the present study, we demonstrated the presence of two distinct mRNA species originating from the common matrilysin gene in the porcine ovary. It is well known that alternative RNA splicing of a single gene is a ubiquitous mechanism for the generation of multiple protein isoforms or tissue-specific gene regulation. Such a alternative splicing has been found in aromatase (Harada, 1992), prolactin receptor (Shirota *et al.*, 1990), and human estrogen sulfotransferase (Bernier *et al.*, 1994) genes. The novel matrilysin mRNA identified in this study should encode a protein whose size is about half that of the wild-type counterpart. The protein seems to have no proteolytic activity because it lacks the catalytic domain essential to the enzyme activity. A structural feature of the

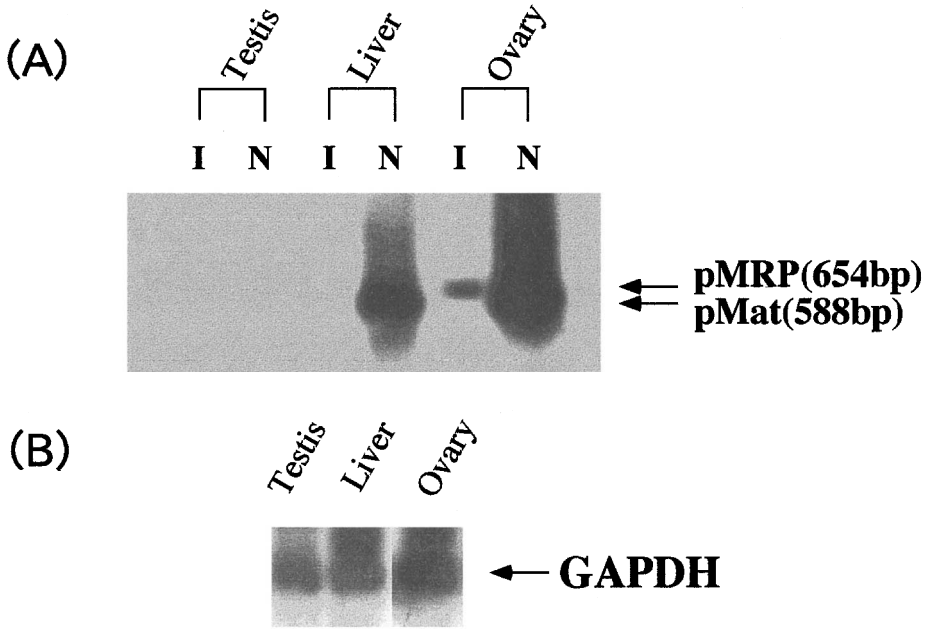

Fig. 5. Detection of the matrilysin-related protein mRNA by RT-PCR. (A) Using poly(A)$^+$RNAs isolated from porcine ovary, testis, and liver, RT-PCR was conducted with a combination of a sense primer S5 and antisense primer AS3 for specific amplification of matrilysin DNA, or S4 and AS3 for the matrilysin-related protein DNA. The products amplified with primers S5/AS3 and primers S4/AS3 were loaded on lane N and lane I, respectively, in a 2.5% agarose gel electrophoresis. (B) Using the same poly(A)$^+$RNAs, RT-PCR was conducted to amplify the DNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a marker protein constitutively expressed in all tissues. Note that equivalent amounts of RNAs were used for the experiment.

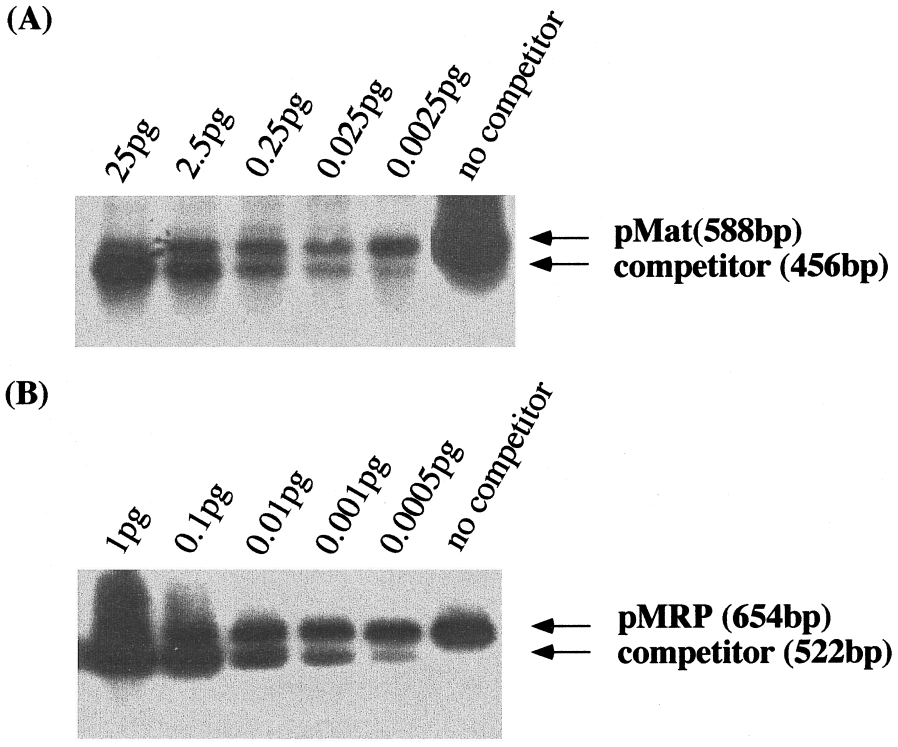

Fig. 6. Competitive RT-PCR of porcine ovary matrilysin and the matrilysin-related protein. The reaction conditions were described in the MATERIALS AND METHODS section. Two PCR primers, S5 and AS3, were used for matrilysin (A), while the primers S4 and AS3 were used for the matrilysin-related protein (B).

putative protein is the presence of a stretch of seven consecutive hydrophobic amino acid residues in its COOH-terminus. However, this hydrophobic segment is apparently too short to serve as a membrane-spanning domain. Therefore, the protein is most likely a secretory protein like the wild-type matrilysin. To the best of our knowledge, this is the first report showing that an unusual matrilysin mRNA is present in mammalian tissues. However, the biological significance of the presence of a COOH-terminal truncated matrilysin in the ovary is not clear at present. To approach this problem, further studies are necessary, including biochemical characterization of the protein and identification of cells responsible for its synthesis. Such studies are currently in progress in our laboratory.

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